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SEX PRESELECTION: HIGH-SPEED FLOW CYTOMETRIC SORTING OF X AND Y SPERM FOR MAXIMUM EFFICIENCY

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ABSTRACT

Sex preselection that is based on flow-cytometric measurement of sperm DNA content to enable sorting of X- from Y-chromosome-bearing sperm has proven reproducible at various locations and with many species at greater than 90% purity. Offspring of the predetermined sex in both domestic animals and human beings have been born using this technology since its introduction in 1989. The method involves treating sperm with the fluorescent dye, Hoechst 33342, which binds to the DNA and then sorting them into X- and Y-bearing-sperm populations with a flow cytometer/cell sorter modified specifically for sperm. Sexed sperm are then used with differing semen delivery routes such as intra-uterine, intra-tubal, artificial insemination (deep-uterine and cervical), in vitro fertilization and embryo transfer, and intra-cytoplasmic sperm injection (ICSI). Offspring produced at all locations using the technology have been morphologically normal and reproductively capable in succeeding generations. With the advent of high-speed cell sorting technology and improved efficiency of sorting by a new sperm orienting nozzle, the efficiency of sexed sperm production is significantly enhanced. This paper describes development of the these technological improvements in the Beltsville Sexing Technology that has brought sexed sperm to a new level of application. Under typical conditions the high-speed sperm sorter with the orienting nozzle (HiSON) results in purities of 90% of X- and Y-bearing sperm at 6 million sperm per h for each population. Taken to its highest performance level, the HiSON has produced X-bearing-sperm populations at 85 to 90% purity in the production of up to 11 million X- bearing-sperm per h of sorting. In addition if one accepts a lower purity (75 to 80%) of X, nearly 20 million sperm can be sorted per h. The latter represents a 30 to 60-fold improvement over the 1989 sorting technology using rabbit sperm. It is anticipated that with instrument refinements the production capacity can be improved even further. The application of the current technology has led to much wider potential for practical usage through conventional and deep-uterine artificial insemination of many species, especially cattle. It also opens the possibility of utilizing sexed sperm for artificial insemination in swine once low-sperm-dose methods are perfected. Sexed sperm on demand has become a reality through the development of the HiSON system.
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Key words: sexed sperm, offspring, flow cytometry, Hoechst 33342, X- and Y-sperm

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INTRODUCTION

Sex preselection has been a long sought goal of the livestock producer. The efficiency of livestock improvement can be greatly enhanced by the utilization of recent advancements in agricultural biotechnology. Interest in this area stems from biblical times and, with recent advances in the technology, it has become a reality. Predetermining the sex of offspring can be done with precision and repeatability using flow cytometric sperm sorting, which is based on measuring the relative difference in DNA content in mammalian sperm and sorting the sperm using a modified flow cytometer/cell sorter (17). The application of the sexing technology to mammals has been proven in several species by the production of offspring (swine, cattle, sheep, rabbit, horse, human) from sexed sperm. The method utilizes the sex-specific difference in sperm DNA for the separation of X- and Y-chromosome-bearing spermatozoa using flow-cytometric sperm sorting (15). Skewed sex ratios have been demonstrated in numerous laboratory and field experiments. Among them are rabbits (15) swine (16, 39), cattle (4, 5, 44, 45), and sheep (6). Offspring of the desired sex have also been born after using the sorting technology in sheep (14, 3). The method also has been adapted to human sperm (21) and in clinical trials has proven successful at producing children of the desired sex (8, 9). All of these results have been produced using the original standard-speed flow sorting of sperm, which produces about 350,000 each of viable X- and of Y-bearing sperm per h (15). On the average, the skewing of the sex ratio in the offspring produced has been about 90% with a range of 75 to 100% of the desired sex. Various reviews of the sexing technology document the progress over the past 10 years in particular (14, 19, 25, 26).

Two aspects of the technology have been substantially altered and have resulted in significant enhancements to the sperm-sexing technology noted above. Sperm sorting for X/Y separation is dependent on the sperm's orientation (17) to the laser beam so as to reduce variability sufficiently to distinguish the small difference in DNA content of the sperm. Due to the compactness of the sperm chromatin, differential fluorescence is exhibited from the edge of the cell compared to the more transparent flat side of the sperm head. This leads to variable DNA fluorescence that masks the small (3 to 4%) X/Y DNA differences of many mammals. A significant enhancement in orientation is gained by the use of a new orienting nozzle system (41, 42) which we have fitted to a high-speed sperm sorter (27). Improvement in orientation has increased from 25% oriented to 70% oriented. This innovation replaces the beveled needle of the original system (17).

The objective of this paper is to chronicle the development of a successful method to preselect the sex of offspring through the use of sperm sorting and to illustrate the improvements that have been made in the original technology. Throughout this paper, in discussing high-speed sorting technology equipped with an orienting nozzle, we are referring to a MoFlo®[a] high-speed cell sorter modified for sperm sorting (17) and equipped with an orienting nozzle (41). Therefore, throughout the paper, the high-speed sperm sorter with orienting nozzle will be referred to as HiSON.

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[a] Cytomation, Inc., Fort Collins, CO, USA

DNA AS A MARKER

Basis For Using DNA Content of Sperm as a Sex-specific Marker Measured by Flow Cytometry

In 1910, Guyer (13) reported the presence of sex chromosomes. One of the first significant attempts to preselect sex was conducted in 1925 by Lush (31) without reference to DNA. That study showed no skewing of the sex ratio in rabbits and pigs based on centrifugation. Several groups carried out research on sex preselection and much of the work in the area was reviewed in a symposium held at Penn State University in 1970 (29).

Once flow cytometry came on the scene in the late 1960's (28), interest was stimulated in measuring DNA in individual cells for many purposes, especially for cancer diagnosis. Another aspect of that early work was that DNA could be a sensitive indicator of mutagenic events associated with various weapons systems being developed or used at that time. Most of the early work on measuring DNA of sperm was done in the United States at the National Laboratories at Los Alamos, NM and at Livermore, CA since that is where the flow-cytometer instrumentation was being pioneered. Gledhill et al. (12) reported the use of flow cytometry for measuring DNA in sperm to determine changes that might occur with genetic damage. Asymmetric shape was shown to cause differential fluorescence. Coupled with random orientation, the differential fluorescence masked the known difference in DNA content between X- and Y-bearing sperm so that the difference could not be measured. It was found that flow-cytometer fluid streams could be changed from cylindrical to flat (10) and when applied to sperm (7) the asymmetrical-shaped sperm could be analyzed with a flow- cytometer/analyzer for DNA content.

In 1979, Moruzzi (35) drew attention to the use of DNA as a potential marker for sex preselection with the publication of data that showed a variety differences between X and Y sperm for numerous species in DNA content measured by differences in chromosome length. An average 6.6% difference in DNA among several species was reported. A high-resolution, orthogonally configured, experimental laser-based flow cytometer that could orient sperm was built (37) and used to show a difference in DNA content in fixed mouse sperm nuclei of 3.2%. The ability to measure a small difference in DNA was confirmed in nuclei of livestock sperm ranging from 3.6 to 4.0% using a simpler analytical but non-sorting flow cytometer (11) based on mercury-lamp excitation. Another symposium (2) served to bring the sex-preselection research up to date and demonstrated not only a broad interest in the area but also updated the potential technologies available to separate X and Y sperm. The knowledge gained over a period of 71 years (1910-1981) contributed to the developmental successes of the most recent 18 years in which DNA was successfully shown to be the only effective marker for separating viable X and Y sperm (15).

SORTING OF SPERM NUCLEI

Development of Sperm Sorting Technology With Standard-Speed Cell Sorters 1983 to 1988

Commercial cell-sorting instrumentation arrived in the 1970's and its performance improved dramatically in 1980 with the development of more advanced data-acquisition equipment. We acquired a cell sorter[b] in 1982 and modified it to sort sperm nuclei (17). This system formed the basis for the current technology as various protocols were developed that led to sorting sperm nuclei of several mammals (18, 26; Figure 1). The sperm used in these studies had been sonicated to remove the tails since tails negatively affected the orientation of the sperm to the laser beam. The utilization of Hoechst 33342[c] a bisbenzimide fluorescent vital dye sensitive to ultraviolet light was found to give improved separation over previous dyes (20). The dye is non-intercalating and binds to the minor groove of the DNA helix. In 1988, Johnson and Clarke (23) showed that sperm nuclei sorted using Hoechst 33342 bound to the DNA were capable of fertilization, indicating that the replicating DNA was functional in the presence of the bound dye and withstood flow-cytometric sorting conditions. Sort-reanalysis was developed to provide continuous validation of the proportion of X- and Y-bearing sperm in a particular semen sample (26) or sorted-sperm population in the laboratory (48).

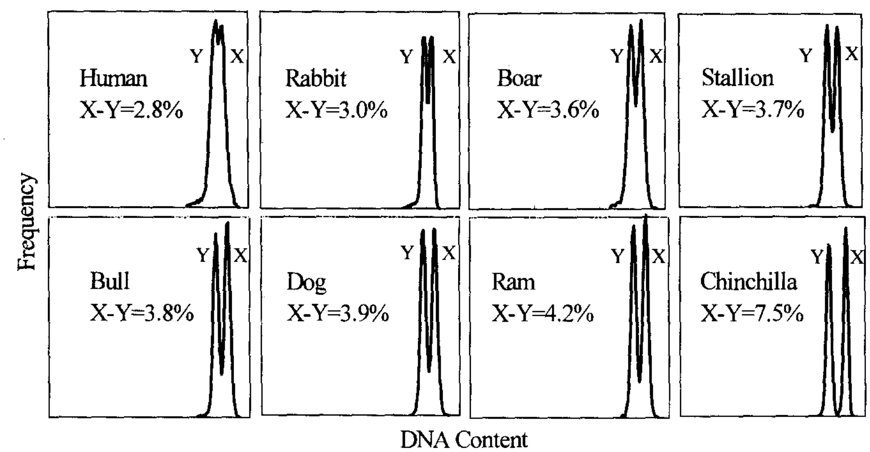

Figure 1. Flow cytometric histograms produced from ejaculated semen from 8 common species illustrating the inherent difference (X-Y) in relative DNA content between X- and Y-chromosome-bearing sperm. The difference in DNA from the 2.8% for human sperm to 7.5% for Chinchilla langier illustrates the difference in DNA associated with chromosome size in domesticated animals and man.

[b] Epics V, Coulter Corporation, Miami, FL, USA

[c] Calbiochem-Behring Corporation, La Jolla, CA, USA

VIABLE SPERM SORTING

Development of Viable Sperm Sorting with Standard Speed Sperm Sorter 1989 to 1991

Hoechst 33342 was found to be highly permeable to the living sperm membrane and that through incubation of the dye/sperm suspension at (32 to 39°C) staining uniformity was enhanced. This finding led to the production of offspring from populations of viable X-bearing sperm and populations of Y-bearing sperm (15). A key factor in the development was the use of a concentrated buffer solution in which to catch the sperm being sorted. As the fluid builds up in the collection tube, sperm swim to the bottom and become accustomed to the more concentrated egg-yolk environment maintaining their viability. A total of ~350,000 sperm could be sorted by this original method in an hour. Rabbits were born with litters showing sex ratios of 94% females and 81% males (15), and litters of pigs were born with 74% females and 68% males (16) all by intra-uterine or intratubal insemination, respectively (Figure 4). Rabbits and pigs were all morphologically normal and were reproductively capable in adulthood showing no negative effects of the sorting process and this was repeated in succeeding generations.

Expansion of Viable Sperm Sorting to Other Livestock and Humans 1992 to 1997

Viable sperm sorting (standard speed) was expanded to collaborations with other groups. A sorting facility established by Animal Biotechnology Cambridge Ltd., UK was used to sort bull sperm for use in conjunction with in vitro fertilization (IVF). Six calves of the correct sex were born from embryos produced from sexed sperm (4). A field demonstration of the capability of the technology (5) was the birth in 1995 of 41 calves, 90% being male, after production and transfer of embryos produced from sorted sperm (Figure 4). Utilization of sexed sperm in the most economical way suggests that freezing sorted sperm would enhance the utility of sexed sperm to the industry. We applied standard freezing technology to bull sperm and found with slight adjustments for concentrating the sperm that we could achieve such a goal. Sorted bull sperm was frozen and thawed and average sperm motility was 30%, and acrosomal integrity, 40%. One and 2 million sperm per 0.5-ml straws were used (19). The first lamb produced by intra-cytoplasmic sperm injection of sexed sperm was reported in 1996(3).

We applied the method to human beings by sorting human sperm (21). Fluorescence in situ hybridization (FISH) was needed to verify the purities of the sorted X- and Y-bearing human sperm due to the small X/Y difference in DNA (2.8%) and inconsistency of sort reanalysis where small DNA differences exist. Clinical trials were initiated and the first child from sorted human X-bearing sperm was born in 1995 to a family that carried the X-linked disease, hydrocephalus. Subsequently, the clinical application of the technology in man has resulted in more than 50 births (8, 9) under the name MicroSort®[d] for human sperm.

Low-dose insemination for cattle using a deep-uterine-insemination technique with sexed sperm was demonstrated through collaboration with Seidel and coworkers in 1997 (44). The technique was used with sexed sperm and provided a useful semen-delivery avenue needed to bring sexed semen to

[d] Genetics and IVF Institute, Fairfax, VA, USA

producers. Semen was brought to Beltsville from Atlantic Breeders Cooperative, Lancaster, sorted into X and Y populations and shipped by air to Colorado where it was inseminated using the deep-uterine technique. A total of $2x10^5$ sperm per dose was sufficient to get pregnancies and produce 17 calves (Figure 4). The average percentage of females from sorted X sperm was 82%.

With improved techniques for pig IVF, sexed sperm were used to produce 2 litters of pigs totaling 10 female piglets (Figure 4). This was the first successful use of sexed sperm to produce sexed offspring from sexed embryos in the pig (39).

Conversion of Standard Speed Sorting to High-Speed Sorting With Orienting Nozzle 1996 to 1999

Achieving gains in sperm throughput as well as efficiency of sorting was necessary for greater applicability of flow-sorted sexed sperm for livestock production using AI. Improving the efficiency of sperm orientation was a key factor since it would increase the number of sperm available for sorting. This effort led to the development of the orienting nozzle described below (41; Figure 2). The second major factor was the commercial development of the high-speed sorter that we were able to acquire and modify. In and of itself, the high-speed sorter with the standard beveled needle (Figure 2) would sort up to 2 million sperm per hour. However, when we adapted the orienting nozzle to the high-speed sorter (HiSON) the capability increased to 6 million sperm of each sex per hour.

CONVERSION TO HIGH SPEED SORTING

Preparation of Sperm for High Speed Sorting (HiSON) Based on DNA Content

Ejaculated semen was collected into a warmed container and transported, maintaining temperature, to the laboratory. Sperm concentration and percentage of motile sperm were determined by standard methods. Proportion of stain per number of sperm is important to maintaining staining uniformity. The semen extender used for maintaining sperm viability and the stain was Beltsville TS (16) for the boar and HEPES-BSA (42) for bull sperm. Most semen extenders appropriate to the species will work for this purpose; however, egg yolk and milk and high levels of BSA as constituents should be avoided since they inhibit uniformity of staining. The staining stock solutions were: Hoechst 33342, (5 mg/mL in water), and food coloring, FD&C #40; 25 mg/mL (22, 27). The food coloring penetrates the membrane of the dead sperm and quenches the intensity of fluorescence of the dead sperm, thus eliminating dead sperm from the viable sperm population. Propidium iodide can serve the same purpose but has the drawback of being an intercalating dye (24).

Ejaculated sperm (150×10^6) were aliquoted into a final volume 1.0 ml of extender that was equilibrated to semen temperature (~35°C). The optimal amount of stain for most species was 40 μg (8 μL of 5 mg/mL stock) Hoechst 33342 per 150×10^6 sperm. Sperm number prepared per mL or per sample tube can be varied as long as the proportion of Hoechst 33342 per sperm is maintained. The sperm-Hoechst suspension was incubated 60 min at 35°C (15) and then moved from the heating block to a styrofoam container maintained at room temperature. To stain dead sperm, 1 μl of FD&C 40 was mixed gently and allowed to sit for 5 min. Samples were filtered through a 30-μ nylon mesh prior to sorting. Sperm are then sorted. In preparing for a long sort (3 h or more), it has been found advantageous to stain new samples of sperm for the second 3 h period.

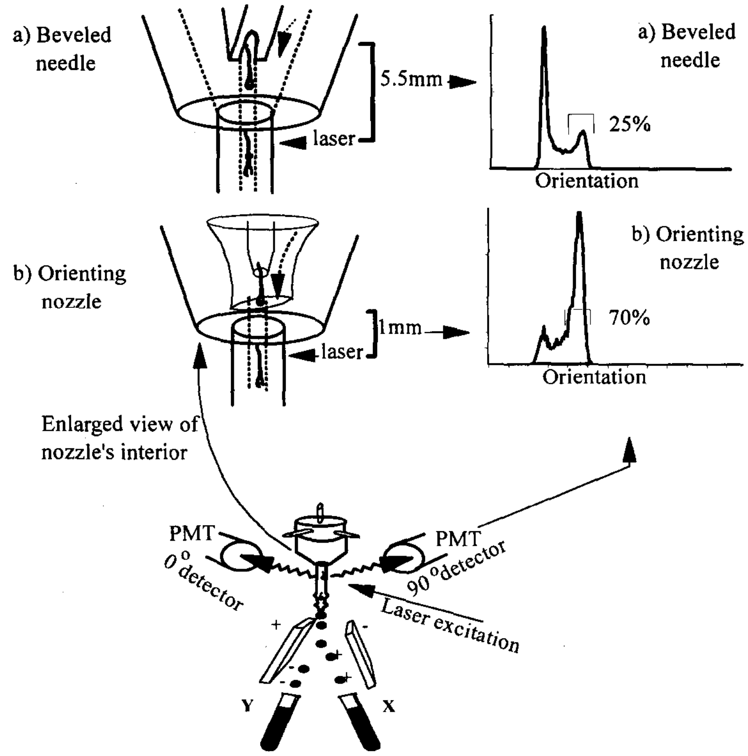

Figure 2. Schematic diagram of basic cell sorter (47) modified for sperm (17). Shown are the additional photomultiplier (PMT) and optical detector in the forward position (0°). The impact of using the Beltsville elliptical nozzle instead of the beveled needle for orienting sperm to the laser beam in a typical modified cell sorter configured for sorting sperm is shown in the two histograms. Because the hydrodynamic forces that orient the stream of fluid are of shorter duration and further away (5.5 mm) from the laser beam when using a beveled needle, sperm lose orientation again before they reach the laser beam. This is compared to the hydrodynamic forces using an orienting nozzle which is subjected to a longer duration of orientation forces and is a shorter distance (1 mm) from laser beam of sperm passing through the orienting nozzle. This results in 2 to 3 times the number of oriented sperm (70% versus 25%) that are available to be sorted because they fall within the gate of the 90°PMT. Increased sorting speed and efficiency in utilizing sperm applied to the high-speed sperm sorter is the result of using the orienting nozzle (b) versus the beveled needle (a). Configuration (b) also requires a sample injection needle, but it can be cylindrical and unmodified. Another modification used with high-speed sorting is the addition of an elliptical beam shaping optic (49) (not illustrated). This beam shaping optic is fitted in the laser path just before the intersection of the laser beam and the sample stream. (Adapted from 41, 27).

Set Up of HiSON for Sorting X- and Y-Chromosome-Bearing Sperm

The high speed sorter equipped with an orienting nozzle (27; Figure 2) is optimally adjusted for sperm sorting using beads[e] and nuclei of sperm from the species to be sorted. System parameters can be adjusted according to the experiment or at the investigator's determination. Our common parameters are: sheath pressure (3.62 kg/cm^2); nozzle with 60-μ orifice; sort deflection=1 drop; 100 mW laser power (351,364 nm); average flow rates from 10,000 to 15,000/sec. To achieve higher throughput we have used flow rates of 30,000 per sec. The lasers are argon-ion lasers that are water cooled[f] operated with ultraviolet optics. A gate is set on oriented sperm (Figure 2) using the 90° fluorescence histogram. The 0° fluorescence histogram displays the DNA content of oriented sperm only, and sort windows are established according to the speed and enrichment of the specific population desired (Figure 3).

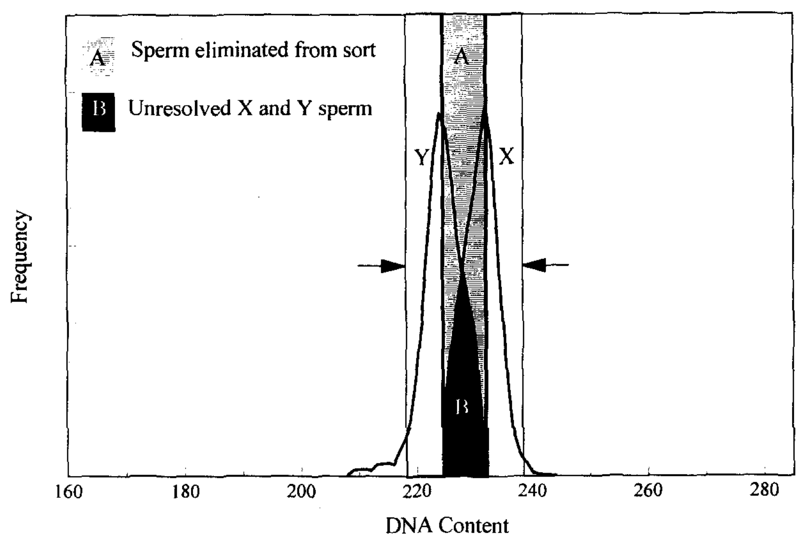

Figure 3. Illustration of a typical histogram of flow-sorting set-up for sperm. Sort windows selecting for X- and Y-chromosome-bearing sperm are based upon the relative DNA content. The outer boundaries (arrows) remain constant for a given species. The interior boundaries when set as shown, to eliminate the area (A) in which X and Y sperm are not resolved (B), can result in X and Y purities near 95%. Sort speeds can be increased at the expense of reducing the purity of X and Y sperm by reducing the number of channels within (A), which always encompasses area (B).

[e] Fluoresbrite BB 4.5μ beads, Polysciences Inc., Warrington, PA, USA

[f] Models 90-5 and 307; Coherent Inc., Palo Alto, CA, USA

Collection of Sorted X- and Y-Chromosome Bearing Sperm

Maintaining sperm viability in high-dilution conditions of cell sorting is dependent on providing a nutrient environment within the collection tube. The procedures described are adapted from the original 1989 (15) method to adjust for high-speed sorting conditions. Tubes used for collecting sorted sperm can be of any size to fit the type of sort being done. A 0.6-mL or 1.5-mL microfuge or 15-mL conical tube have worked effectively, the former for short sorts, the latter for sorts of longer duration. To reduce sperm loss through adhesion, tubes should be precoated by filling with a 1% BSA solution for 1 h prior to use to neutralize any charge on the tube surface. A volume of 0.05 to 0.5 mL of TEST-yolk (2 or 20%) should be added to the coated tubes, depending on size of tube. Generally a minimum of 50 µl of TEST-yolk added to the smaller tubes and 0.5 mL in the larger tubes is satisfactory to provide a concentrated haven for the sorted sperm. Two percent TEST-yolk is effective to collect sorted sperm for IVF (39, 30), while up to 20% works for sorted sperm to be used for insemination. Sorted sperm were centrifuged from 350 x g to 700 x g for 10 to 15 min, depending on size of tube. The supernatant was aspirated and the pellet rediluted according to the particular usage. TEST-yolk extender has been very effective for most species. However, other extenders using egg yolk to give a concentrated environment can also be used. Validation of sorted sperm populations were conducted using "sort reanalysis" as described (48).

<u>Evaluation of X and Y sorted sperm membranes</u>. Sperm quality should be monitored once sperm have been sorted using standard assessment techniques. Sperm motility and live/dead sperm evaluations are helpful to test for the ability of the sperm to fertilize ova. Membrane changes do take place during the process. Chlortetracycline has been used to delineate the membrane effects of sorting in vitro (32). The primary conclusion to be drawn from numerous studies is that a higher proportion of membranes are acrosome reacted or pre-capacitated in sperm that have been sorted (33). This is one reason sorted sperm do not need to be capacitated before they are used for IVF. Addition of seminal plasma to the staining buffer as well as the collection medium appears to be beneficial. However, these findings have been shown in vitro and await in vivo testing (33).

<u>Swimming velocity of X and Y sperm</u>. For more than 25 years there has been controversy surrounding the question of whether or not Y sperm swim faster than X sperm due to their slightly lower weight. Although the conclusions were drawn relative to human sperm, it is likely that sperm of other species would respond similarly should the hypothesis be true. We conducted a study to test the hypothesis using computer-assisted motion analysis (CASA). There was no difference with respect to the swimming velocity between Y sperm and X sperm of the bovine (36) as measured by CASA.

Performance Parameters Under Routine Sorting Conditions With HiSON

Assuming a flow rate of 14,000/sec with sperm that were 80% motile at the outset, one could expect the following output: About 1400 to 1700 sperm/sec each of X and of Y would be sorted with a resulting purity of X or Y at approximately 90%. Losses to the sort are the dead sperm, and the misoriented sperm. Assuming an orientation rate of 70% and a sort-window elimination of 30% (mixed population of X and Y sperm) there is a final yield of about 20 to 30% of the total sperm with which one started (10 to 15% for each population of X- or Y-bearing sperm). Many factors affect flow integrity including doublets, large aggregates, staining uniformity, resolution, sperm viability, orientation, and coiled tails (43). It was also established that, as sperm pass through the flow cell of the sperm sorter, they go either head first or tail first with equal frequency (43). No data have been produced to determine if there is an advantage of head or tail first in the integrity of the sort. Placement of sort windows is also a factor in the outcome. Various parameters can be adjusted for given sorts that would affect flow rate, sort rate, production of sorted sperm per h as well as fertilization. Table 1 shows parameters that will produce 6 million sperm per h in each direction (6 million X and 6 million Y sperm) for a 1-h sort total of 12 million sperm. Sorting parameters are shown that were used to obtain 6 million sperm per h at 90% purity. These are average results that on any given day can be influenced by the factors mentioned above to increase the production by 20% or reduce the production rate by 20%.

Table 1. Mean values for boar sperm sorts using a HiSON and operating at 3.16 kg/cm^2 over 2.5 h at conditions described in the text illustrating a routine setup and sorting experiment (N=4)

Flow rate		Sort rate		No. of sperm sorted	Sort reanalysis[a]	
Sperm/sec	% Oriented	Sperm/sec	Sperm/h		% X	% Y
14,000	65.1	1,700	6×10^6	15×10^6	91	89

[a] Sort reanalysis was performed to determine purity using a 300-µl volume aliquoted from each sorted sample (48).

Table 2 illustrates the potential production using higher flow rates for 11 million X sperm/h at 87% purity using the HiSON. Increasing the flow rate to 30,000 sperm/sec and utilizing a higher sheath pressure (4.22 kg/cm^2) and droplet formation of 100 kHz while increasing the size of the orientation gate for X-bearing-sperm can yield X sorts of over 10 million per h with 85 to 90% purity.

Table 2. Mean values for boar-sperm sorts using HiSON and operating at 4.22 kg/cm^2 over 1.0 h at 100 kHz droplet formation(N=3)

	X Sort rate		% X Sort	Y Sort rate		%Y Sort reanalysis
Flow rate/sec	per sec	per h	reanalysis	per sec	per h	
30,000	3,150	11.3×10^6	87	2,000	7.2×10^6	77

[a] Sort reanalysis to determine purity was performed using a 300-µl volume aliquoted from the sorted sample (48).

Adjustment of parameters such as this are possible to achieve a specialized outcome, i.e. only one population. The sorting of X sperm alone is the easiest due to the fact that it carries the most DNA and, therefore, is the brightest in response to the exciter-laser beam, which places it on the upper end of the histogram. Consequently, the sorting of X sperm at higher speeds results in a purer sample than can be obtained with Y sperm. This is due to the fact that slightly misanalyzed X sperm will fall into the Y-sort window.

Data to illustrate the near upper limits of the HiSON system in terms of viable intact sperm production rate with the current optics and configuration are shown in Table 3. Through the use of sort reanalysis the proportionate purity of X sperm was determined at various rates. Three sorts of viable sperm from each of three boars were done (N=9). The increased production rate of X sperm was accomplished by including more of the sperm in the X sort window (Figure 3) which includes more of area B in the sorted X population, and thus results in a reduction of the purity of X sperm. In comparison to Table 2, it should be noted that a lower Khz droplet formation is used in table 3 (75 Khz) versus 100 Khz as well as a lower pressure (3.92 versus 4.22 kg/cm^2). More optimum sorting conditions were obtained in the lower pressures and the lower Khz settings.

Table 3. Comparison of various collection rates of flow sorted sperm and the effect on purity of the sorted X population of sperm

	Number of sperm sorted per h with respective % purity of X sperm					
Boar	9×10^6	10.8×10^6	12.6×10^6	14.4×10^6	16.2×10^6	18×10^6
1	86	86	82	80	77	76
2	83	83	81	78	79	73
3	83	90	81	81	78	71
Mean	84	86	81	80	78	73

[a] Sort reanalysis to determine purity was performed using a 300-µl volume aliquoted from the sorted sample (48). Flow rates averaged 24,000 sperm/sec at 75 Khz and 3.92 kg/cm^2.

Table 4 shows the data from sorts at the highest pressure that we have used for sorting (4.22 kg/cm^2). There was a 10% increase in DAR in the sorted sperm illustrating a mild effect of HiSON sorting conditions on the integrity of the acrosomal membranes of boar sperm.

Differences in Sperm DNA Among Species Affects the Sorting of Sperm

The most critical differences in sorting procedures relative to species are the differences in DNA between X and Y sperm within a species. For example the Chinchilla langier has a 7.5% difference (Figure 1) making it relatively easy to get a pure sort (98 to 100%) since the bimodal split (Figure 1) is nearly complete. The common species of domestic animals are different in X-Y DNA in varying degrees: rabbit (3.0%), pig (3.6%), bull (3.8%), horse (3.7%), and sheep

(4.2%). The narrower the DNA difference, the more care is required in setting the sort windows. Most domestic animals mentioned have the paddle shaped sperm with a clear difference between edge and flat side.

Table 4. Comparison of fresh control semen with sorted sperm from the boar to determine the effect of high speed sort conditions on acrosomal integrity.[a]

	Acrosomal Integrity (%)			
	NAR	DAR	MAR	LAC
Control	96.9	2.3	0.4	0.3
Sorted	87.9	10.5	0.7	1.1

[a] Boar sperm were stained and sorted under 4.22 kg/cm^2 pressure; control boar sperm were not stained and not sorted. Sperm were fixed in glutaraldehyde and evaluated for acrosomal integrity. A small volume was sorted (50 µl) onto 2% Test-Yolk buffer (5 µl) and immediately fixed with equal volume of 2% glutaraldehyde (38). Equivalent numbers of control sperm were pipetted and similarly fixed. N=22 observations and 5 boars. Normal Apical Ridge (NAR); Damaged Apical Ridge (DAR); Missing Apical Ridge (MAR) and Loose Acrosomal Cap (LAC).

Man, on the other hand, has sperm that are smaller than domestic animal sperm and are shaped less like a paddle but more angular or lobular. However, differential fluorescence is also characteristic of human sperm allowing their selection according to their orientation to the laser beam. The sperm of man has a DNA difference of 2.8% (21), requiring exact tuning of the sperm sorter and refining sperm sorting procedures. Also it is particularly difficult to get a Y sort that is more than 80% pure using human sperm (8, 21). We have not attempted to sort sperm of species with an X/Y difference of less than 2.8%.

Sheath and Staining Buffer Systems for Sperm Sorting

The sheath fluid needs to be an electrolyte solution such as saline, PBS, TRIS or clear extender with electrolyte properties. We use PBS effectively with the addition of 0.1% BSA (w/v) and 0.1% EDTA (w/v). EDTA is used as a constituent for sorting boar sperm to enhance membrane stability. The BSA is present to help reduce agglutination. Balanced salt solutions of different types work well. Semen extenders for staining solutions vary with species. We have used Tris for rabbit (15), HEPES and TALP for bull semen (14) and BTS for boar semen (16). Most semen-buffer systems can be adapted to sperm staining and sorting. Presence of egg yolk in staining buffers frequently introduces more difficulty with uniform staining. Depending on the purpose for which the sperm are being sorted, it may be advantageous to use the same buffer as a sheath fluid as the final sorted sperm extension buffer.

Impact of Laser Power On Sorted-Sperm Recovery, Fertilization and Embryo Recovery

Since the inception of our research on sexing viable sperm (15), wattage of the laser has been chosen on the dual premise that the lower the laser output power the less effect upon the fertilization and embryo development, and the higher the laser power the less difficulty in getting and maintaining a stable bimodal histogram for sorting. It has also been clear that the effects of laser output power cannot be viewed alone. This is because the fluorochrome Hoechst 33342 bound to the minor groove of the adenine-thymine regions of the DNA helix, is excited by the laser light. Early in our studies with living sperm (15, 16), we used 150 to 200 mW of laser power. Additional studies (19) also were done using a standard-speed sperm sorting system (Epics V/753). We have looked at the direct impact of laser power on rabbit embryonic development (34) and found that 75 mW using the Epics optics has minimal impact on fertilization rate and embryonic development while increasing levels of laser output power lower rates of fertilization and embryonic development. In recent experimentation in swine with the HiSON, we compared a laser output powers of 25 mW and 125 mW. Sows were surgically inseminated and embryos were flushed 44 h later. In this preliminary experiment, 125 mW did not affect fertilization rate or embryonic development, but 25 mW increased the percentage of unfertilized eggs and underdeveloped embryos[g]. This result is somewhat unexpected based on our original premise that lower laser power would be less damaging; however, it may simply reflect that incomplete laser excitation with the bound stain is detrimental to developmental competence of the embryo. Studies are underway to define the role of Hoechst 33342 more clearly in embryonic development as there may be an effect of the fluorochrome alone (30, 33).

Sperm Orientation: Efficiency and Impact on Sorted Viable Sperm Production

Three aspects of instrumentation are especially critical to sort sperm efficiently for use in various semen-delivery situations (e.g. AI, Deep AI, IVF, Intra-uterine insemination (IUI), Intra-tubal insemination (ITI) and ICSI). The first of these is the need for a forward-fluorescence detector to replace the normative-light-scatter detector present on most cell sorters. This detector placed in the 0° position allows one to gate off of the normative 90° fluorescent detector and read analytically the 0°detector for DNA content (Figure 2). Gates are placed around the brightest population (90°) because this is the population that has the edge (brightest fluorescence) directed to the 90° detector. Removing the variability associated with sperm that are not properly oriented allows CV's of 1% for each population and production of bimodal peaks (Figures 2 and 3) that can be fitted with sorting windows. A second instrumental factor is the nozzle through which one can control the presentation of individual sperm to the laser beam and, thus, the 90° detector. The "orienting nozzle" (Figure 2; 17) has proved to be much more efficient than the beveled needle (27, 42). The nozzle is more effective because the sperm leaving the elliptical chamber are oriented ~1 mm from intersecting the laser beam, while with the beveled needle, the sperm are exiting the needle oriented ~5.5 mm from the laser-intersect point. Orientation at a point that is more than 4 times closer to the laser beam increases efficiency 2 to 3 times greater (15) for viable sperm. The third instrumental factor that has boosted sorting efficiency is the adaptation of the orienting nozzle to high-speed cell sorters. In our laboratory, we adapted the

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[g] LA Johnson, HD Guthrie, GR Welch, Unpublished data.

orienting nozzle to the high-speed sorter as indicated for sperm sorting. With these 3 improvements in instrumentation, a production rate 6 million per h of each of X- and Y-sperm fractions is achieved for routine conditions. Higher rates of sorting with the HiSON also have been achieved (Tables 1, 2 and 3) with the configuration in Figure 2. An additional configuration item that may affect the high rates are the beam shaping optics (BSO) that we inserted into the laser path prior to intersection of the laser beam and sample stream. These elliptical optics are unmodified from those used in the Epics V flow cytometer systems. However, optimizing these optics for sperm may be advantageous to gain higher sorting rates.

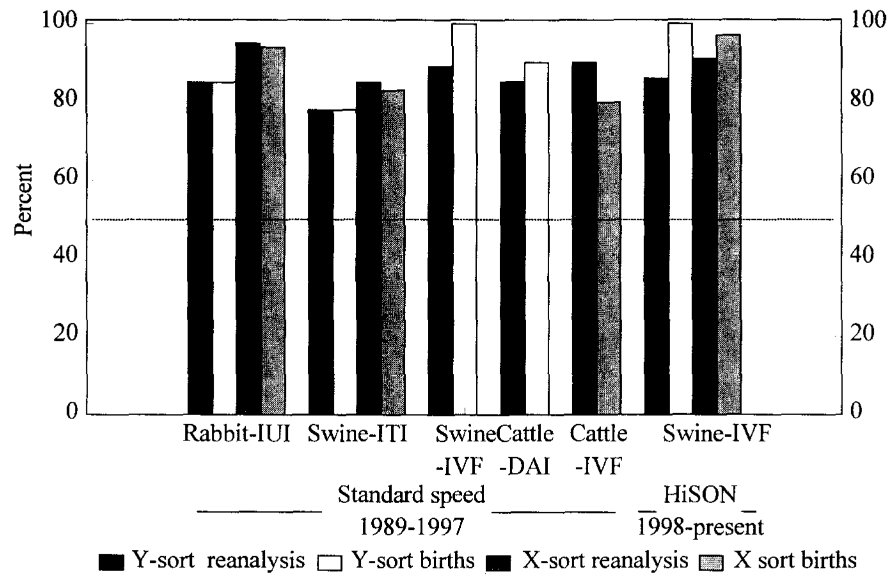

Figure 4. Comparison of skewing of the sex ratios based on sort reanalysis and sex of offspring with sperm from various species using two different sorting technologies (standard speed, Epics V/753);[h] and (a high-speed, MoFlo) equipped with an orienting nozzle. The bar graph illustrates the consistency of skewing. The term DAI=deep uterine insemination. Overall, the bars represent approximately 400 offspring. Respective references in which data are reported are: Rabbit (15); Swine (16, 39); Cattle AI (44); Cattle IVF (4, 5); Swine IVF (1, 40).

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[h] Coulter Corporation, Miami, FL, USA

FERTILIZATION WITH SORTED X AND Y SPERM

Effects of HiSON Conditions on Blastocyst Development in Swine

In studies designed to compare the effects of Hoechst 33342 and sorting conditions, it was found that monospermic penetration was not affected. However, polyspermic penetration of pig ova was significantly reduced in stained-unsorted sperm. Cleavage rates proved to be greater in control sperm but were essentially equal in the sorted and stained-unsorted groups. When HiSON sperm-sorter pressures were compared, there was no difference between 1.76, 2.81 and 4.22 kg/cm^2 in terms of fertilization rate (30). Early attempts to sort sperm using a high-speed sorter with a system pressure of 6.43 kg/cm^2 were detrimental to sperm viability[i].

Fertility of Sexed Sperm After Sorting Using HiSON

Studies have been conducted using sexed sperm after high-speed sorting for IVF in the pig (Figure 4). In two of these studies, sperm were sorted using the system described above, and the sperm were used for insemination of ova in vitro. Previous studies in our laboratory had produced the first piglets from sexed sperm sorted by the original protocol using the standard-speed sorter. Those studies produced two IVF litters of pigs, which were 100% female (Figure 4). Improvements in IVF protocols led us to try to repeat this work but with the HiSON and using ova matured in vitro rather than in vivo. One study (40) was conducted in close proximity to the sperm sorter at Beltsville; 9 litters of piglets were born from transferred embryos. Three were controls producing 23 pigs; 52% were male and 48% were female. In the treatment group, 34 pigs were born from 6 sows; 33 were females and one was a male (97%). Ova inseminations occurred within 1 h of completing the sorting process, and embryos were transferred at the 4-cell stage. In another study (1), 8 litters were born at the University of Missouri after sexed sperm, sorted in Beltsville, had been shipped by air, held overnight and used approximately 20 h post sort for fertilization of in-vitro-matured pig ova. In this collaboration, 3 litters were born from embryos produced from Y-sorted sperm and comprising 9 male piglets. Five litters were born from transferred embryos produced from X-sorted sperm resulting in 24 piglets, of which 23 were female (97%). Litter size in the 2 studies were 5.8 for those fertilized within 1 h of sort and 4.2 for those fertilized 20 h post sort. These overall results demonstrate the effectiveness of using sexed sperm to produce sexed offspring in the pig by first producing sexed embryos.

High-speed sorting of bull sperm can provide sexed sperm for AI in the conventional manner. We inseminated cows into the body of the uterus under standard AI conditions with sperm from a 1-h sort. This was effectively 2 to 3 million motile sexed sperm. Pregnancies were obtained and calves born of the predicted sex. HiSON offers the opportunity to use conventional AI in cattle as well as by deep-uterine insemination (22).

Cryopreservation of sorted sperm extends the utility of the sperm for application for most species. In cattle, Seidel and colleagues (45, 46) demonstrate the effectiveness of frozen, sorted sperm. This work indicates that frozen sorted sperm will be a major means of applying the

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[i] LA Johnson, D Pinkel, unpublished, data.

technology in practice. Freezing sorted sperm from swine, on the other hand, is more problematic in that the boar sperm membrane is more easily damaged by freezing than is the case for bull sperm. We have recently had some success in getting fertilized eggs after inseminating frozen sexed boar sperm intratubally[j]. Freezing sorted boar sperm reduces its fertility to the some degree as it does non-sorted sperm.

Results at Other Locations Using High Speed Sperm Sorting

Extensive field trials using high-speed technology essentially as described here have been conducted by Seidel and colleagues in collaboration with XY, Inc. over the past couple of years. One thousand heifers have been inseminated with sexed sperm and 370 heifers have been inseminated with control sperm (45). Accuracy of producing a male or female as determined by the sorted sperm that was inseminated approached 90%. Pregnancy rates were within 90% of the unsexed controls. Uterine body insemination was nearly as successful as insemination bilaterally into the uterine horns. The results of these studies with cattle illustrate the effectiveness of using uterine insemination and conventional AI, making the application of sexed sperm in the cattle industry using high-speed sperm sorting technology likely. Furthermore, the use of frozen semen in addition to fresh semen using the sorting technology adds an important commercial dimension to the process, since frozen semen is highly important to widespread use of the technology in the cattle industry.

CONCLUSIONS

The Beltsville Technology was successfully converted from the standard-speed sorter. The description in the foregoing pages represents the current state of the art in flow-cytometric sorting for separation of X- and Y-chromosome-bearing sperm of mammals. The use of a high speed sorter modified for sorting sperm and adapted with an orienting nozzle is capable of producing up to 11 million sperm per/h of X sperm in our laboratory with 85 to 90% purity. More routine sorting can produce 6 million sperm/h of each sex where both X and Y sperm are needed. Additionally, even faster sorting rates (18 million/h) can be achieved albeit, at a lower purity of X sperm (75%). It is anticipated that further refinements of current optical technology may increase the purity achieved at high sorting rate. It is anticipated that sexed sperm will be marketed in the very near future. The utilization of the HiSON technology is essential to widespread application of the that technology. Refinement of the HiSON system is likely to lead to even greater production rates of X and Y sperm. Pressures under which sorting takes place do not appear to affect sperm membranes or fertilization significantly. The HiSON technology is adaptable to both animal and human sperm systems.

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[j] LA Johnson, WMC Maxwell, D Guthrie, GR Welch, W Garrett, unpublished, data.

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